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The Anticancer Effects of Vitamin E Derivative alpha-TEA in Human Hematological Malignancies

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The Anticancer Effects of Vitamin E Derivative alpha-TEA in Human Hematological Malignancies

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Abstract

The Anticancer Effects of Vitamin E Derivative alpha-TEA in Human Hematological Malignancies

By

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α-TEA (α-tocopherol ether linked acetic acid) has been shown to induce apoptosis in human prostate, ovarian and breast cancer cells in culture and in xenograft models by promoting pro-apoptotic pathways and inhibiting anti-apoptotic pathways. Studies investigated the ability of α-TEA to induce apoptosis in human hematological malignant cell lines Jurkat, Raji and U266, representing T cell leukemia, B cell lymphoma and multiple myeloma, respectively. The three cell lines were cultured in the presence of different concentrations of α-TEA for different time periods, and examined for apoptosis

by annexin V – FITC analyses, DAPI staining, and western blotting for poly (ADP-ribose) polymerase cleavage. α-TEA induced apoptosis in all three cell lines in a dose and time dependent manner. Levels of pro-apoptotic molecules DR5, c-Jun N-terminal protein kinase (JNK), C/EBP homologous protein (CHOP), caspase 9, and caspase 3 were upregulated in α-TEA treated cells in comparison to vehicle controls. Caspase 8 was activated in Jurkat and U266 cells but not in Raji cells. Apoptosis and pro-death signaling mediators were blocked by ceramide inhibitor, desipramine. The anti-apoptotic nuclear factor kappa B (NF-κB) signaling pathway was down-regulated in α-TEA treated Raji and U266 cells. Combinations of omega-3 fatty acid docosahexaenoic (DHA) and α-TEA significantly enhanced apoptosis in Jurkat cells in comparison to single treatments and vehicle control. In summary, α-TEA induced apoptosis in the malignant hematological cell lines is via shared and distinct pathways. ASMase/ceramide-mediated JNK activation and endoplasmic reticulum (ER) stress mitochondrial dependent apoptosis are involved in α-TEA induced apoptosis in the three cell lines; however, the cell lines exhibit cell type-specific responses to α-TEA: activation of death receptor/caspase 8 pathway is involved in Jurkat cells, suppression of NF-κB signaling is involved in Raji cells, and the U266 cells share both of these pathways for the induction of apoptosis.

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Chapter 1: Introduction

Vitamin E refers to a group of eight structurally distinct forms divided into tocopherols (α, β, γ, δ) and tocotrienols (α, β, γ, δ)–tocotrienols (Gropper et al., 2004). The tocopherol α-form is membrane associated and exhibits antioxidant properties (Gropper et al., 2004). Epidemiological, cell culture, and preclinical studies show that tocopherols, other than the α form, and tocotrienols exhibit anticancer properties (Constantinou et al., 2008). Finding that the anticancer effects of vitamin E forms are disassociated with their antioxidant effects suggests that the structure of these compounds may be accountable for their anticancer effects. Based on this thought, to develop more potent anticancer agents, researchers have modified the structure of vitamin E forms and synthesized vitamin E analogs. Two examples of analogs of α-Tocopherol which possess anticancer properties are described: vitamin E α-tocopheryl succinate (VES) is synthesized by adding a succinate moiety to carbon 6 on the chroman head of α-tocopherol by an ester linage. VES possesses anti-proliferative activity in epithelial cancers such as breast cancer and colon cancer, and has no toxic effects in normal cells (Kline et al., 2004). However, in the body VES is hydrolyzed by cellular esterases into α-tocopherol and succinate, both of which have no detectable anticancer effects (Weber et al., 2002). To explore a more clinical relevant vitamin E analog for cancer treatment, our laboratory synthesized the RRR-α-tocopherol ether linked acetic acid (alpha-TEA), by attaching an acetic acid moiety to the chromanol head of α-tocopherol molecule by a nonhydrolyzable ether linage (Lawson et al., 2003). Both *in vitro* and *in vivo* studies have shown that α-TEA has selective antitumor effects in a variety of cancers (Kline et al., 2004; Jia et al., 2008). α-TEA induces apoptosis in cancer cells but not in normal epithelial cells (Lawson et al., 2003).

Studies in our lab have shown that α-TEA induced apoptosis in human breast, ovarian and prostate cancer cells (Snyder et al., 2008; Anderson et al., 2004; Jia et al., 2008). The mechanisms by which these apoptotic effects are mediated involve upregulation of proapoptotic gene expression and donnregulation of antiapoptotic gene expression (Yu et al., 2010; Jia et al., 2008; Yu et al., 2006). The anticancer effects of α-TEA in hematological malignant cells have not previously been investigated.

Docosahexaenoic acid (DHA, C22:6n-3) is a type of omega 3 (n-3) polyunsaturated fatty acids. Epidemiological and *in vitro* studies have shown that DHA has anticancer effects in a wide range of cancers, including breast cancer (Jude et al., 2006), colon cancer (Chapkin et al., 2008), prostate cancer (Shaikh et al., 2008), melanoma (Albino et al., 2000) and leukemia (Siddiqui et al., 2001). The anticancer effects of DHA have been shown to be mediated by apoptosis and cell cycle arrest (Shaikh et al., 2008; Siddiqui et al., 2003).

Hematological malignancies include a large group of heterogeneous neoplasms originating from bone marrow or lymphatic tissue (Rodriguez-Abreu et al., 2007). These diseases can be broadly divided to leukemia, lymphoma and myeloma (Rodriguez-Abreu et al., 2007). According to SEER statistics, from 2003-2007, the age-adjusted incidence rates for leukemia, lymphoma and myeloma are 12.3, 22.4, and 5.6 per 100,000 men and women per year, respectively (Altekruse et al., 2010). The age-adjusted death rates are 7.2, 7.3, and 5.6 per 100,000 men and women per year, for leukemia, lymphoma and myeloma, respectively (Altekruse et al., 2010). Among leukemia cases diagnosed from 2003-2007, about 10.9% were under age 20. For lymphoma, only 3% were diagnosed under age 20 and none of myeloma cases were diagnosed under age 20 (Altekruse et al., 2010). Although survival outcomes from hematological malignancies have improved

during the past decades due to early detection and improved treatments, cancer relapse remains a problem. Patients with certain types of leukemia, non-Hodgkin's lymphoma or multiple myeloma eventually relapse and the prognosis is poor. Therefore, novel agents with potent anticancer properties and reduced or no toxicity are needed. Although the anticancer effects of α-TEA have been intensively examined in human epithelial cancers, until now, this promising anti-cancer agent has not been investigated in human hematological cancers.

In this study, (i) α-TEA was shown to induce cell death by apoptosis in human Jurkat (T cell leukemia), Raji (B cell lymphoma), and U266 (multiple myeloma) cell lines. (ii) α-TEA induced apoptosis in the three cell lines via common and distinct pro-death and anti-apoptotic signaling mediators. (iii) α-TEA in combination with DHA significantly induced apoptosis in the Jurkat cell line in comparison to single treatments and control.

Chapter 2: Literature Review

VITAMIN E

Vitamin E is a group of eight fat-soluble forms called tocopherols and tocotrienols that are richly contained in plant oils such as wheat germ oil, sunflower, and safflower (Eitenmiller, 1997; Murphy et al., 1990). Each of the eight forms is composed of a phenolic functional group on a chroman ring and a phytyl tail. Tocopherols and tocotrienols are distinguished based on whether the phytyl tail is saturated or unsaturated, respectively. Tocopherols and tocotrienols are classified as α, β, δ, γ, determined by the different number and position of methyl groups on the chromanol ring (Figure 1) (Gropper et al., 2004). RRR-α-tocopherol is the most abundant form in the human body, and γ-tocopherol is the most abundant form in the American diet (Burton et al., 1998; Jiang et al., 2001). In the human body, RRR-α-tocopherol is mostly found in adipose tissue; whereas, it is localized to membranes in cells (Gropper et al., 2004).

The best known physiological function of RRR-α-tocopherol is to maintain plasma membrane integrity by preventing oxidation (Gropper et al., 2004). The hydroxyl group on carbon 6 on the chromane ring of RRR-α-tocopherol denotes the hydrogen needed to reduce oxidized molecules, such as polyunsaturated fatty acids (Gropper et al., 2004). Clinical studies show RRR-α-tocopherol to reduce the risk of cardiovascular disease by preventing oxidation of low density lipoproteins which contribute to the accumulation of lipids and macrophages on the blood vessel walls (Jialal et al., 1992; Mosca et al., 1997). Other functions of different vitamin E forms include suppression of cholesterol synthesis and tumor growth (Gropper et al., 2004).

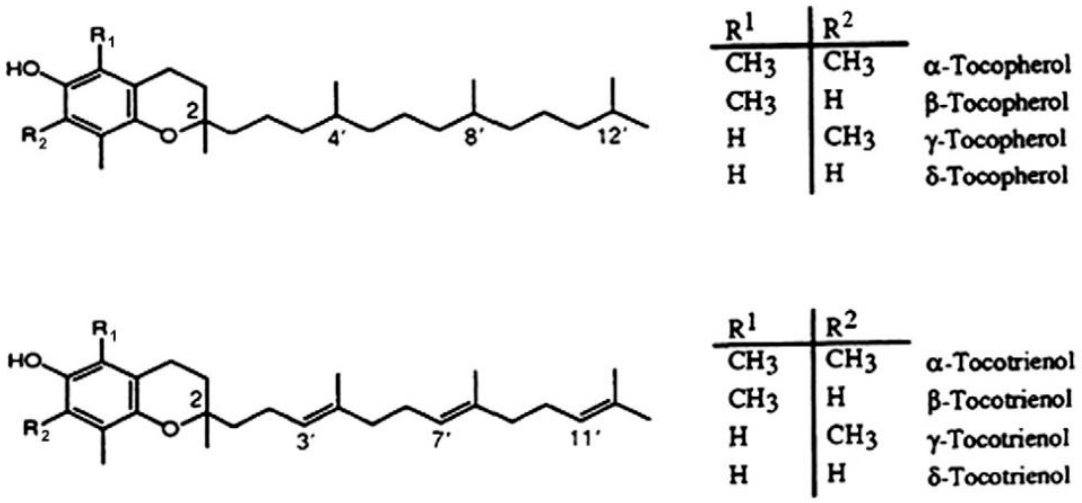

Figure 1. Structures of vitamin E compounds. (Source: Nesaretnam, 2008)

The tumor suppression effects of tocopherols and tocotrienols

Epidemiological studies have shown that some forms of tocopherols and tocotrienols have protective effects against prostate cancer, colon cancer and rectal cancer by preventing the occurrence of cancers and suppressing tumor promotion (Wright et al., 2007; Weinstein et al., 2005; Kune and Watson, 2006). The anticancer abilities of tocopherols and tocotrienols differ. Tocotrienols have stronger anticancer activity than tocopherols (Constantinou et al., 2008). Within the same form of vitamin E, γ- and δ-forms have stronger anticancer ability than α- and β- forms (Constantinou et al., 2008). Although α-tocopherol has the greatest antioxidant activity among vitamin E forms, its anticancer property is the least. This fact suggests that the antioxidant ability and anticancer activity of vitamin E forms are not associated, and the minor difference in structure between vitamin E forms may be accountable for the different anticancer effects of these compounds (Constantinou et al., 2008).

Vitamin E Derivatives

The structure of RRR-α-tocopherol has been modified in an effort to pursue more potent anticancer agents. One example is vitamin E α-tocopheryl succinate (VES), which is synthesized by adding a succinate moiety to carbon 6 on the chroman head of RRR-α-tocopherol by an ester linage (Figure 2) (Prasad et al., 2003). VES suppresses cell proliferation and induces apoptosis in a variety cancers (Neuzil et al., 1999; Yu et al., 2002; Yu et al., 2003); however, a major disadvantage of VES is that when administered in vivo the ester linkage is hydrolyzed by esterases in the body, releasing α-tocohperol and succinate, neither has anticancer effects (Weber et al., 2002).

To explore a more clinical relevant vitamin E analog in cancer treatment, our laboratory synthesized the RRR-α-tocoperol ether linked acetic acid analog (alpha-TEA), by attaching an acetic acid moiety to carbon 6 on the chroman head of RRR-α-tocopherol by a nonhydrolyzable ether linage (Figure 2) (Lawson et al., 2003). Both *in vitro* and *in vivo* studies have shown that α-TEA has selective antitumor effects in a variety of cancers (Kline et al., 2004; Jia et al., 2008). *In vitro* studies have shown that α-TEA induces apoptosis in a variety of human epithelial tumors (breast, prostate, ovary, colon and lung cancers) and lymphoblastic malignancies (leukemia and lymphoma) (Lawson et al., 2003). Pre-clinical animal studies have shown that α-TEA reduces tumor burden and metastasis in mouse xenograft tumor models (Lawson et al., 2003; Lawson et al., 2004; Zhang et al., 2004; Jia et al., 2008; Anderson et al., 2004; Riedel et al., 2008). Compared to VES, α-TEA shows more potent anticancer activity, probably due to the nonhydrolyzable ether linkage that attaches the acetic acid moiety to the chroman head (Lawson et al., 2004; Anderson et al., 2004). Equally important, α-TEA shows no detectable cytotoxic effect in normal epithelial cells and tissues (Lawson et al., 2003). These properties of α-TEA make this compound a promising anticancer agent.

The mechanisms of the anticancer effects of α-TEA have been explored in human breast, prostate and ovary cancer cells, which include enhancing pro-apoptotic cellular pathways and suppressing anti-apoptotic pathways. α-TEA upregulates protein levels of death receptors Fas and DR5 and induces activation of caspase 8, 9 and 3 (Yu et al., 2006; Jia et al., 2008; Yu et al., 2010). α-TEA also induced phosphorylation of c-Jun N-terminal kinase (JNK) which may induce apoptosis through mitochondria or via endoplasmic reticulum (ER) stress (Yu et al., 2006, Jia et al., 2008; Tiwary et al., 2010). α-TEA suppresses pro-survival signaling mediators such as PI3K/AKT, Ras and ERK, and downregulates protein levels of anti-apoptotic factors caspase 8 homologue FLICE-inhibitory protein (cFLIP), survivin and Bcl-2 (Jia et al., 2008; Yu et al., 2006; Snyder et al., 2008; Shun et al., 2010).

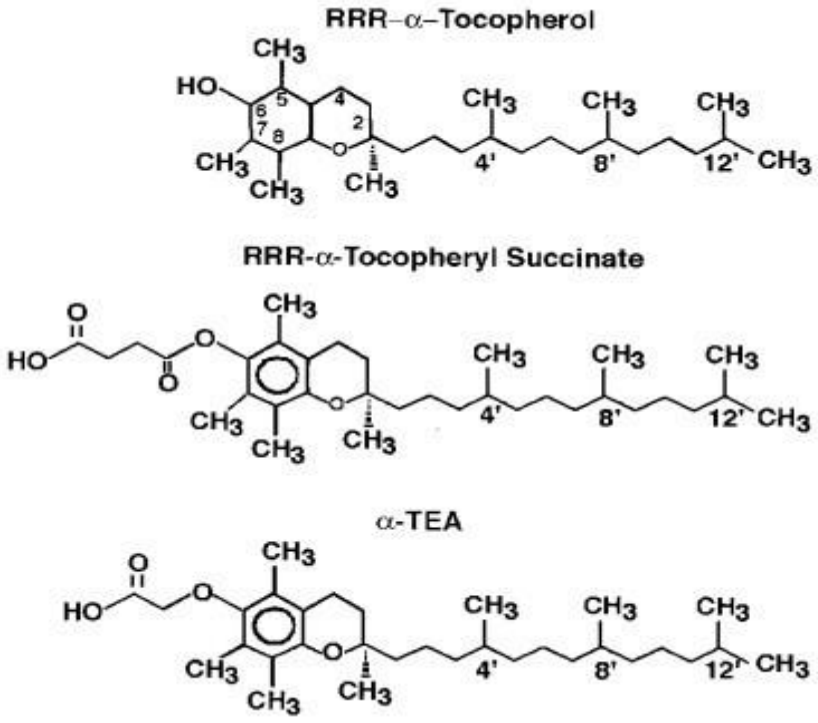

Figure 2. Comparison in structures between α-tocopherol, VES and α-TEA. (Source: Lawson et al., 2003)

APOPTOSIS

Apoptosis, also known as programmed cell death, is a form of cell death that differs from necrosis. Compared with necrosis, the process of apoptosis is strictly controlled and does not cause inflammatory reactions that could damage surrounding cells. Apoptosis was initially characterized by its morphological changes which involve condensation of nucleus and cytoplasm, fragmentation of nucleus and formation of apoptotic bodies (Kerr et al., 1972).

Apoptosis has important physiological significance. It is a mechanism for normal development, elimination of abnormal cells and maintenance of tissue homeostasis. Apoptosis is executed by a class of cysteine aspartyl-specific proteases, known as

caspases (Nicholson 1999). In response to certain intracellular or extracellular stimuli, initiation caspases are cleaved and activated. Activated caspases further cleave each other to amplify the death signaling. Finally the executioner caspases are activated and cleave cellular substrate, such as proteins and chromosomes (Creagh et al., 2003). Apoptotic cells are typically removed by phagocytes (Kerr et al., 1972). The membrane phospholipid phosphatidylserine that is normally located on the inner side of cell membrane is exposed on the surface of cell membranes and functions as a recognition signal by phagocytes (Fadok et al., 1992).

Pathways Mediating Apoptosis

It is well known that apoptosis can be induced through two pathways: extrinsic pathway and intrinsic pathway (Taylor et al., 2008). In the extrinsic pathway, the ligation of death receptors on the cell membrane triggers trimerization of death receptors and recruitment of the adapter protein Fas-associated death domain protein (FADD) and caspase 8, forming the death-inducing signaling complex (DISC). Caspase 8 is thereby cleaved and activated, which in turn activates a series of downstream caspases including the direct apoptosis executioner caspase 3, leading to apoptosis. The intrinsic apoptosis pathway is dependent on mitochondria. Apoptotic signaling inside the cell triggers loss of mitochondria transmembrane potential, causing release of cytochrome *c* from mitochondria intermembrane. Cytochrome *c* together with apoptotic protease activating factor-1 (APAF1) and caspase 9 forms a complex called apoptosome. Activated caspase 9 activates downstream caspases including caspase 3 which triggers apoptosis. There is cross talk between the extrinsic and intrinsic apoptotic pathways. Activated caspase 8 can cleave and activate Bid which translocates to mitochondria and activates mitochondria dependent apoptosis (Taylor et al., 2008).

Regulation of Apoptosis

Apoptosis is strictly controlled by multiple regulatory factors. A key regulation factor of apoptosis is p53. The p53 protein functions as a transcription factor. Under normal conditions, p53 expression is kept low. In response to stress, p53 expression is elevated and thereby induces apoptosis by upregulating the expression of pro-apoptotic genes such as Fas (Müller et al., 1998), DR5 (Wu et al., 1997), BAX (Miyashita, Reed, 1995), PUMA (Nakano, Vousden, 2001), and NOXA (Oda et al., 2000). Apoptosis is inhibited by Bcl-2 family proteins. Bcl-2 family proteins include anti-apoptotic proteins Bcl-2, Bcl-X_L and pro-apoptotic proteins (BAX, Bak, Bid, Bad, Bim, NOXA, PUMA) (Igney, Krammer, 2002). These proteins control the integrity of mitochondria membrane. Activated BAX and Bak induce mitochondrial membrane transition leading to release of cytochrome *c (*Igney, Krammer, 2002). Bcl-2 and Bcl-$_{XL}$ inhibit activation of BAX and Bak (Igney, Krammer, 2002). Other regulators of apoptosis are the inhibitors of apoptosis (IAP) family proteins: XIAP, cIAP1, cIAP2, NAIP, ML-IAP, ILP2, KIAP, apollon and survivin (Deveraux, Reed, 1999). These proteins act as caspase inhibitors by mediating caspases degradation (Deveraux, Reed, 1999). Moreover, cFLIP is an inhibitor of caspase 8. cFLIP has similar structure as caspase 8, but missing the catalytic site, so that it competes with caspase 8 to bind to DISC and prevent activation of caspase 8 (Krueger et al., 2001).

CERAMIDE AND APOPTOSIS

Ceramide is a sphingolipid that acts as a lipid second messenger (Kolesnick, 2002). Ceramide plays an important role in the induction of apoptosis and is therefore called tumor suppressor lipid (Hannun, 1997). Ceramide can be produced by de novo synthesis, hydrolysis of sphingomyelin by the enzyme sphingomyelinase (SMase) or salvage pathway (Jenkins et al., 2009). There are three types of sphingomyelinase, acid

(aSMase), neutral (nSMase) and alkaline sphingomyelinase depending on the optimum pH conditions for the enzymes to work. Ceramide may be located in the caveolae or rafts in plasma membrane, lysosomes, mitochondria or endoplasmic reticulum (Mimeault, 2002).

Ceramide triggers apoptosis in response to various stresses such as transmembrane receptor ligation, UV radiation and chemical stress (Chen et al., 2008) . It has been suggested that ceramide may induce apoptosis via multiple mechanisms, including directly acting on mitochondria (Taha et al., 2006). Published data show that ceramide increases permeability of both inner and outer mitochondria membrane, promoting release of cytochrome *c* from mitochondria (Siskind et al., 2002; Novgorodov et al., 2005). Plasma membrane ceramide may trigger apoptosis by inducing Fas oligomerization, raft coalescence and caspase 8 activation (Mimeault, 2002). In addition, ceramide may mediate apoptosis by signaling transduction (Taha et al., 2006). Moreover, ceramide is thought to induce caspase-independent apoptosis (Taha et al., 2006).

ENDOPLASMIC RETICULUM STRESS AND APOPTOSIS

Endoplasmic reticulum (ER) is a subcellular organelle that is responsible for manipulating and transporting secretory proteins, and is also a site of Ca^{2+} storage in the cell (Hussain, Ramaiah, 2007). ER stress occurs when unfolded proteins accumulate in the ER (Xu et al., 2005). Stimuli that can induce ER stress include glucose deprivation, viral infection and conditions disrupting protein degradation (Xu et al., 2005). ER stress initially activates adaptive mechanisms to restore ER to normal status by promoting protein degradation and reducing protein accumulation in ER. Prolonged or excessive ER stress, however, induces apoptosis (Xu et al., 2005).

ER stress induces apoptosis via numerous mechanisms (Kim et al., 2008). One mechanism is through JNK-mitochondria by Ask1 (Kim et al., 2008). In ER stress, Ask1 is activated by Ire1, causing activation of JNK. Activated JNK mediates mitochondria-dependent apoptosis by activating the proapoptotic protein Bim and inhibiting the antiapoptotic protein Bcl-2 (Putcha et al., 2003; Yamamoto et al., 1999). Another mechanism is through inducing expression of transcription factor C/EBP homologous protein (CHOP). CHOP expression is induced in ER stress by ATF4, ATF6, or XBP-1 (Kim et al., 2008). Elevated CHOP may induce apoptosis by inhibiting expression of Bcl-2 or inducing expression of DR5 (McCullough et al., 2001; Yamaguchi, Wang, 2004). Moreover, Ca^{2+} released from ER during ER stress plays a critical role in apoptosis induction by multiple mechanisms (Breckenridge et al., 2003). Ca^{2+} can directly increase mitochondria permeability by flowing into mitochondria (Kroemer, Reed, 2000). Ca^{2+} released from ER also activates Ca^{2+} dependent proteases calpain which activates Bax and Bid and inhibiting Bcl-2 and Bcl-X_L by direct cleavage (Rizzuto et al., 2003; Breckenridge et al., 2003).

APOPTOSIS AND CANCER

Resistance to apoptosis is a hallmark of cancer (Hanahan, Weinberg, 2000). The mechanisms of apoptosis resistance in cancer cells involve increased anti-apoptotic signaling and decreased pro-apoptotic signaling (Igney, Krammer, 2002). Aberrant elevated PI3K/AKT and constitutively activated NF-κB signaling are often found in tumor cells (Igney, Krammer, 2002). In addition, anti-apoptotic molecules such as Bcl-2, Bcl-X_L, IAP proteins, and cFLIP are often overexpressed in tumor cells (Igney, Krammer, 2002). On the other hand, pro-apoptotic signaling is attenuated in tumors. For example, the central apoptosis regulator p53 gene is mutated or deleted in half numbers

of tumors (Harris, 1996). Moreover, malfunction of death receptors is also found in some tumors (Igney, Krammer, 2002). Novel molecular treatments that target these altered signaling mediators are under intense investigation and are promising cancer therapeutic strategies (Nicholson, 2000).

HEMATOLOGICAL MALIGNANCIES

Hematological malignancies are neoplasm happened in lymphatic system or bone marrow. These diseases can be roughly classified as leukemia, lymphoma and plasma cell neoplasms (Rodriguez-Abreu et al., 2007). The estimated new cases of hematopoietic malignancies in the United States in 2009 are 139,860, accounting for about 9% of all newly diagnosed cancers, and the estimated deaths from these cancers are 53,240 (American Cancer Society, 2009).

Leukemia

Leukemia originates from blood and bone marrow. Leukemia can be divided to four major types: acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML). Leukemia is the most common cancer in children aged from 0 to 14, accounting for 32.7% of all childhood cancers and causing about one third of cancer-related deaths in children (American Cancer Society, 2009). However, leukemia is found more often in adults than in children. According to SEER report, from 2003 to 2007, the median age at leukemia diagnosis is 66 years old (Altekruse et al., 2010). Although the outcome of leukemia in children is good with survival rate about 80-85%, the outcome in adult leukemia patients is poor and leukemia in most patients will relapse (Larson, Stock, 2008). The 3 year survival rate for adult ALL is only 30-40% (Rowe et al., 2005).

For acute leukemia, patients usually are given chemotherapy followed by consolidation chemotherapy or autologous/allogeneic stem cell transplantation depending on age and prognostic factors of patients (Shipley, Butera, 2009). Immunological therapy is a relatively new strategy that is under intense development (Shipley, Butera, 2009). For chronic leukemia, treatments are usually given in advanced diseases. For early stage diseases, treatment is given only when disease progresses, otherwise a "watch and wait" monitoring strategy is applied (Hallek et al., 2005).

New agents targeting specific genetic alterations in tumor cells have been developed, including multidrug resistance inhibitors, proteosome inhibitors, antiangiogenesis agents, tyrosine kinase inhibitors, and apoptosis inhibitors (Tallman, 2005). Such agents hold promise for improvement of outcome in patients with poor prognostic factors, older patients who cannot tolerate intense chemotherapy, and relapsed/refractory cases.

Lymphoma

Lymphomas are neoplasms originating from immune systems including both lymphoid tissues and extranodal organs. According to the pathological characteristics lymphoma can be divided into Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL). The majority of lymphoma cases are NHL, accounting for 88.6% of lymphoma new cases in 2009 (American Cancer Society, 2009). The Hodgkin's lymphoma is identified by the presence of Reed-Sternberg cells (Mastasar, Zelenetz, 2008). Treatments of early stage lymphoma include radiotherapy, chemotherapy, or combined radiotherapy and chemotherapy. Intensive chemotherapy and autologous or allogeneic stem cell transplantation are used for relapsed or refractory patients (Mastasar, Zelenetz, 2008). Immunotherapy is under active development for the treatment of lymphoma. The most

intensively studied immunotherapeutic agent is rituximab, a monoclonal antibody against the general B cell marker CD20, which may be used alone or in combination with chemotherapy or stem cell transplantation (Mawardi et al., 2009). Clinical studies have shown that rituximab improve survival rates in relapsed and refractory patients (Van Oers, et al., 2006). Classical HL is considered a curable disease. Most HL patients treated with radiotherapy or combined radiotherapy and chemotherapy have very good outcome with a 5 year relative survival rate of 86.8% for cases diagnosed in 1999-2006 (Altekruse, 2010). Compared to HL, NHL has relatively poor outcome with 5 year relative rate of 69.1% in 1999-2006 (Altekruse, 2010).

Anti-apoptotic molecules including cFLIP and XIAP have been found to be responsible for apoptosis resistance and pathogenesis of HL (Evens, et al., 2008), making these genes targets of potential therapeutic strategies.

Myeloma

Multiple myeloma (MM) is a B cell (antibody secreting plasma cell) malignancy characterized by accumulation of malignant plasma cells in the bone marrow. MM is the second most common hematological malignancy after non-Hodgkin lymphoma in the United States (Raab et al., 2009). Estimated new cases and deaths in 2009 in the United States are 20,580 and 10,580, respectively (American Cancer Society, 2009). MM is more common in African Americans than in whites. Traditional therapy for this disease is chemotherapy followed by stem cell transplantation for patients younger than 65 or continuous chemotherapy for those not eligible for stem cell transplantation (Kyle, Rajkumar, 2008). Patients usually have a short time remission followed by relapse (Schwartz, Vozniak, 2008). With intense drug development during the past several years, novel agents are now being used in the clinic, i.e., Bortezomib, Thalidomide and

Lenalidomide (Laubach et al., 2009). Because of these advances, the outcome of multiple myeloma has improved. However, multiple myeloma is still an incurable disease and the five year survival rate is only around 34% (Brenner et al., 2008). Moreover, side effects of current therapeutic agents remain a problem.

CANCER CELL LINES

Three cell lines were used in this study: Jurkat, Raji and U266. The Jurkat cell line is derived from the peripheral blood of a 14-year-old male patient with T-lineage ALL (Schneider et al., 1977). The Raji cell line is derived from an 11-year-old male patient with Burkitt's lymphoma, a B cell origin non-Hodgkin's lymphoma, in 1963 (Pulvertaft, 1964). The U266 cell line is derived from the peripheral blood of a 53-year-old male patient with multiple myeloma (Nilsson et al., 1970). U266 cells produce interleukin-6 (IL-6) and secrete large amounts of IgE.

Chapter 3: Materials and Methods

CHEMICALS AND REAGENTS

α-TEA was prepared as previously described (Lawson et al., 2003). A 40 μM stock solution was made by dissolving α-TEA in ethanol. DHA and desipramine were purchased from Sigma-Aldrich (St. Louis, MO). A 100 mM stock solution of DHA was made with DMSO and stored. A 50 mM stock solution of desipramine was solubilized with DMSO and stored. All stock solutions were stored at -20°C. Chemicals were diluted with medium before treatment.

Primary antibodies used for western blotting include: PARP (Santa Cruz), caspase 8 (Cell Signaling), caspase 9 (Cell Signaling), caspase 3 (Santa Cruz), Bid (BD Pharmingen), Fas (Santa Cruz), DR5(Cell Signaling), p-JNK(Cell Signaling), JNK(Santa Cruz), p-cJun(Santa Cruz), CHOP (Santa Cruz), p-IκBα (Cell Signaling), IκBα (Santa Cruz), cyclinD1 (Santa Cruz), XIAP (Cell Signaling), cFLIP (Santa Cruz), survivin (Santa Cruz), Bcl-2 (Santa Cruz), and GAPDH (Cell Signaling).

CELL CULTURE

Human acute lymphoblastic leukemia cell line Jurkat, and Human Burkitt's lymphoma cell line Raji, available in our lab, were cultured in RPMI 1640 medium (GIBCO), supplemented with 10% fetal bovine serum (FBS) (GIBCO), 200 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO), and 4.5 g/L glucose (Sigma). Human myeloma cell line U266 was purchased from ATCC, Manassas, VA. U266 cells were cultured in RPMI 1640 medium supplemented with 15% FBS, 200 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in 37°C incubator with humidity and 5% CO_2. Cells were plated at a density of 5×10^5 cells/ml in 2% FBS supplemented medium before treatment.

DAPI STAINING FOR APOPTOSIS EVALUATION

Apoptosis was evaluated by staining the nucleus with 4', 6'-diamidino-2-phenylindole (DAPI). DAPI strongly binds to nucleic acid in live and dead cells, allowing observation of nuclear morphological change during apoptosis. DAPI (Roche Diagnostics, Indianapolis, IN) was diluted with methanol. Treated cells were collected, washed with PBS, and incubated with DAPI solution (5 μg/ml) in methanol at 37°C for 5 minutes. Cells were then observed under a fluorescent microscope (Olympus IX71). Cells containing condensed or fragmented nuclei were counted as apoptotic cells. For each sample, at least 300 cells were counted. Data were expressed as percentage of apoptotic cells to total counted cells.

ANNEXIN V- FITC FLOW CYTOMETRY ANALYSIS

Apoptosis was also evaluated by annexin V-FITC/propidium iodide (PI) flow cytometry analysis. The protein annexin V binds to phosphatidylserine which is externalized of plasma membrane during early stage of apoptosis, thus allowing detection of apoptotic cells by measuring the fluorescein conjugated with annexin V. PI is a dye which cannot penetrate plasma membrane of viable cells but can easily enter dead or late stage apoptotic cells to bind to DNA. Cells were plated at 5×10^5 cells/ml in 2% FBS medium and cultured overnight. Cells were then treated with α-TEA or DHA for different time points. Treated cells were collected, washed with ice cold PBS twice, resuspended in annexin V-FITC (Invitrogen, Carlsbad, CA) in binding buffer (BD Biosciences Parmingen, San Diego, CA) and incubated at room temperature for 15 minutes. Right before measurement, PI (Invitrogen) solution (1:1000 diluted in PBS) was added. The C6 flow cytometer® (Accuri Cytometers, Inc. Ann Arbor, MI) was used to measure the fluorescence. CFlow® software (Accuri Cytometers, Inc. Ann Arbor, MI)

was used to analyze data. Data were expressed by percentage of annexin V-FITC positive to total cells.

WESTERN BLOT ANALYSIS

Treated cells were collected by centrifuge at 1400 rpm for 4 minutes and washed once with PBS. Cell pellets were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris, 0.5% deoxycholate, pH 7.4) and kept on ice for 20 minutes. Cell lysates were vortexed and centrifuged at 13,000 rpm for 10 minutes at 4°C. Supernatants containing cellular proteins were used for western blot analysis. Cellular proteins were quantified using Bio-Rad protein assay (Bio-Rad, Hercules, CA). 20 μg protein was resolved by SDS-PAGE and transferred to nitrocellulose membranes (Optitran BA-S supported nitrocellulose). Membranes were blocked by 5% nonfat dried milk in 1 × TBST (20mM Tris, 150mM NaCl, 0.1% Tween-20, pH 7.6) at room temperature for 30 minutes, and then incubated with primary antibodies 1:1000 diluted in 0.1% BSA solution in TBST overnight at 4°C. Membranes were washed with TBST for 5 minutes and then incubated with horseradish peroxidase conjugated goat-anti-mouse or goat-anti-rabbit secondary antibodies (Jackson Immunoresearch, Rockford, IL) 1:1000 or 1:2000 diluted in 5% milk in TBST at room temperature for 35 minutes. After 3 times of wash with TBST protein bands were visualized by enhanced chemiluminescence (Perkin Elmer, Waltham, MA). Densitometric analysis of protein bands was performed using ImageJ software (http://rsbweb.nih.gov/ij).

REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

The XBP-1 mRNA splice variants were detected using Reverse Transcription PCR (RT-PCR). Treated cells were collected and washed with PBS. Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) following

manufacturer's instruction. RT-PCR was performed using SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen, Carlsbad, CA). β-actin mRNA levels were also measured as loading control. The primers used to detect XBP-1 were:

5'-AAACAGAGTAGCAGCTCAGACTGC-3'(forward) and

5'-TCCTTCTGGGTAGACCTCTGGGAG-3'(reverse).

The primers used to detect β-actin were:

5'-GGCGGCACCACCATGTACCCT-3'(forward) and

5'-AGGGCCGGACTCGTCATACT-3'(reverse).

PCR products were subject to DNA electrophoresis.

STATISTICAL ANALYSIS

Student's t-test was performed to determine significant difference between control and treatments. $P < 0.05$ was considered as statistically significant.

Chapter 4: Results

α-TEA Induced Apoptosis in Human Hematological Malignant Cells

The pro-apoptotic effect of α-TEA in human hematological malignant cells was evaluated by DAPI nucleus staining. Human T cell leukemia (Jurkat), B cell lymphoma (Raji) and multiple myeloma (U266) cells were plated at 5×10^5 cells/ml in reduced serum (2%) medium and allowed to grow overnight. The three cell types were treated with vehicle or α-TEA for 24 hours at their exponential growth phase. DAPI staining of cells treated with α-TEA showed condensed chromatin and fragmented nuclei which is characteristic of apoptotic cells (Figure 3).

α-TEA induction of apoptosis in these cell types was determined by annexin V-FITC/PI analyses. The three cell types were treated with 5, 10 and 20 μM α-TEA or vehicle for 24 hours (Fig 4A). Jurkat cells were the most sensitive with α-TEA significantly inducing apoptosis in Jurkat (5 μM), U266 (10 μM), and Raji (20 μM) in comparison to controls (Fig 4A). Jurkat, Raji and U266 cells were treated with 10, 20 and 10 μM of α-TEA, respectively, for 8, 16, and 24 hours. Significantly enhanced levels of apoptosis were detected in Jurkat, Raji, and U266 cells at 16, 8, and 16 hours, respectively (Fig 4B).

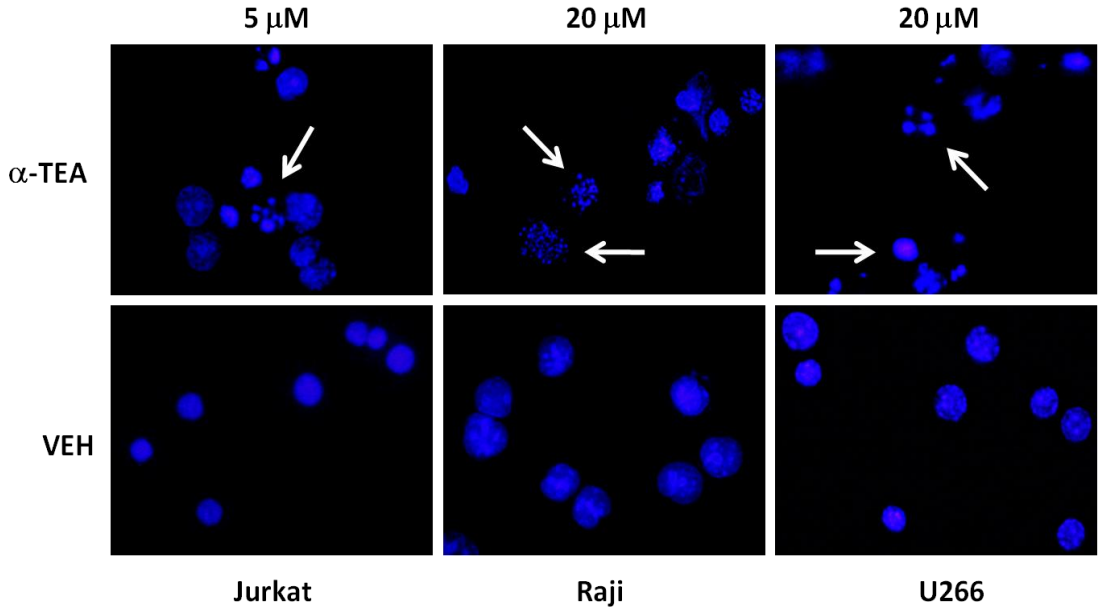

Figure 3. Pro-apoptotic effect of α-TEA in Jurkat, Raji and U266 cells as evaluated by DAPI nuclei staining. Jurkat, Raji and U266 cells were treated with vehicle or α-TEA 5 μM (Jurkat) or 20 μM (Raji and U266) for 24 hours. Cells were collected and stained with DAPI. Apoptosis was indicated by nuclear fragmentation and shrinkage. Data are representative of three independent experiments.

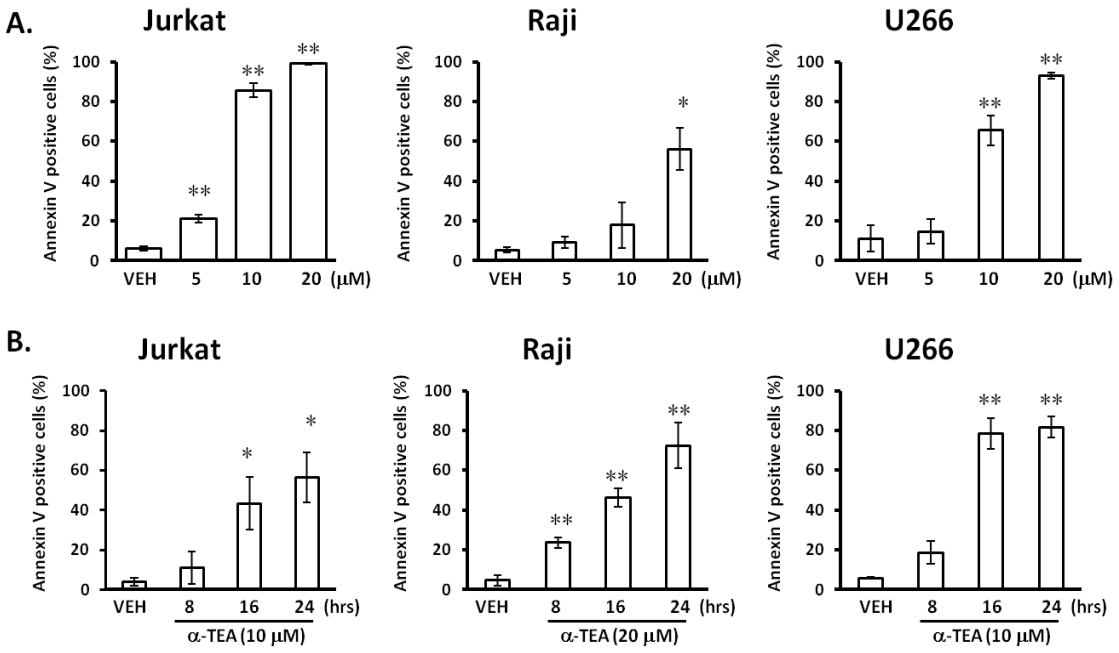

Figure 4. Pro-apoptotic effect of α-TEA in Jurkat, Raji and U266 cells as evaluated by annexin V-FITC/PI analyses. (A) Cells were treated with vehicle or α-TEA (5, 10, or 20 μM) for 24 hours. **(B)** Cells were treated with vehicle or α-TEA (10 or 20 μM) for 8, 16, or 24 hours. Apoptosis was evaluated by annexin V-FITC/PI analysis. Data are presented as mean ± SD of three independent experiments. Student's *t* test was used to determine the statistical significance. *: $P < 0.05$ relative to vehiclecontrol. **: $P < 0.01$ relative to vehicle treatment.

α-TEA INDUCED ACTIVATION OF MOLECULES IN APOPTOTIC PATHWAYS

Western blot analyses were conducted to determine apoptotic pathway mediators induced by α-TEA. Jurkat, Raji and U266 cells were treated with 10, 20 or 15 μM of α-TEA, respectively, or vehicle for various time periods. Whole cell lysates were analyzed by western blots analysis. Caspase 3 substrate, PARP (apoptosis indicator), was cleaved in all the three cell lines in response to α-TEA treatment. Caspases 9 and 3 were activated (cleaved) in all three cell lines. Caspase 8 and Bid activation (tBid) were observed in Jurkat and U266 cells, but not in Raji cells. These data suggest that α-TEA induced apoptosis in Jurkat and U266 cells is caspase 8 dependent and that Bid cleavage activates caspase 9. Where as, in Raji cells α-TEA induced apoptosis is caspase 8 independent (Fig 5).

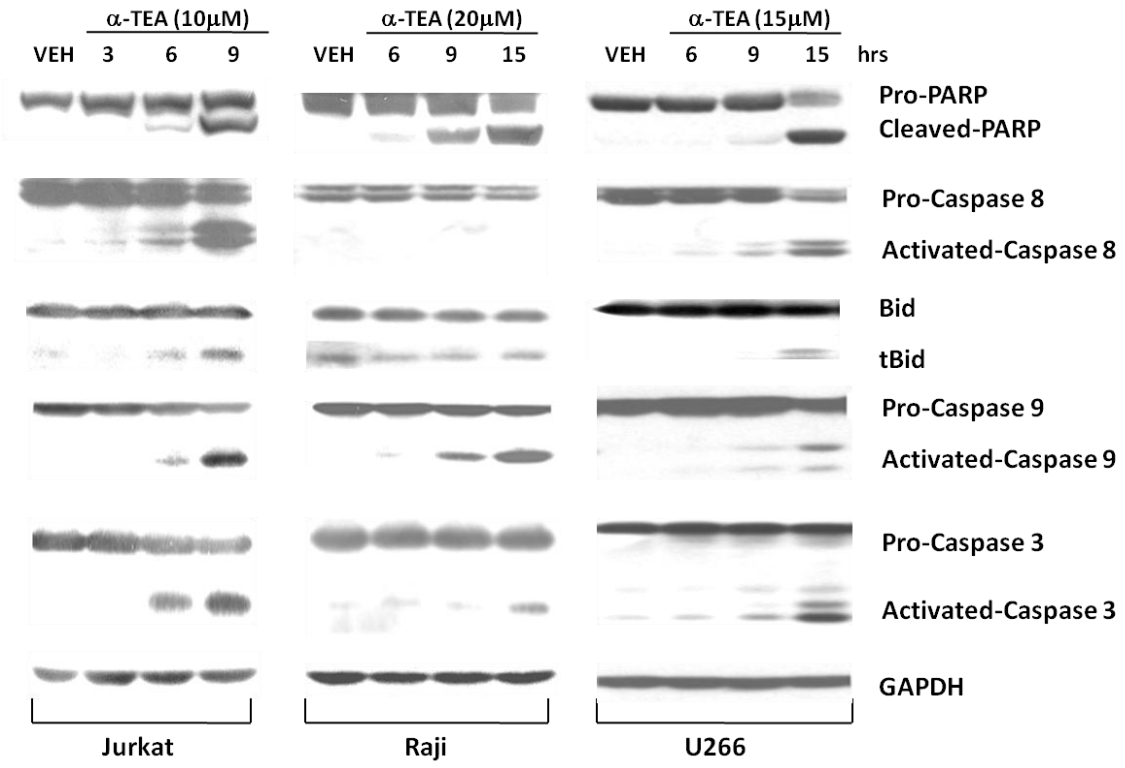

Figure 5. Signaling mediators involved in α-TEA induced apoptosis in Jurkat, Raji and U266 cells. Jurkat, Raji and U266 cells were treated with vehicle or α-TEA at indicated doses for various time periods. Whole cell lysates were analyzed for protein levels of cleaved PARP, caspases 8, 9, and 3, Bid and tBid by western blotting. GAPDH was used as a loading control. Data are representative of three independent experiments.

α-TEA UPREGULATED DEATH RECEPTORS, PHOSPHO-JNK AND INDUCED ENDOPLASMIC RETICULUM (ER) STRESS IN JURKAT, RAJI AND U266 CELLS

To further explore the upstream signaling mediators that trigger the apoptotic pathways, protein levels of JNK and CHOP were examined by western blot analysis (Figure 6A). Jurkat, Raji and U266 cells were treated with α-TEA or vehicle for various time periods and whole cell lysates were analysed by western blotting. Protein levels of death receptor DR5 were upregulated in all three cell lines; whereas, elevated Fas levels were observed in Jurkat and Raji cells but not in U266 cells. These data show that death receptors are involved in α-TEA induced apoptosis in Jurkat and U266 cells. For Raji cells, since caspase 8 activation was not observed, the role of Fas and DR5 upregulation needs further identification. Furthermore, levels of phospho-JNK2/1, cJun, and CHOP were upregulated in all three cell lines (Figure 6A). Splicing of XBP-1 mRNA was induced by α-TEA (Figure 6B), further showing that ER stress is induced in response to α-TEA treatment in the three cell lines, and that ER stress is involved in α-TEA induced apoptosis (Figure 6B).

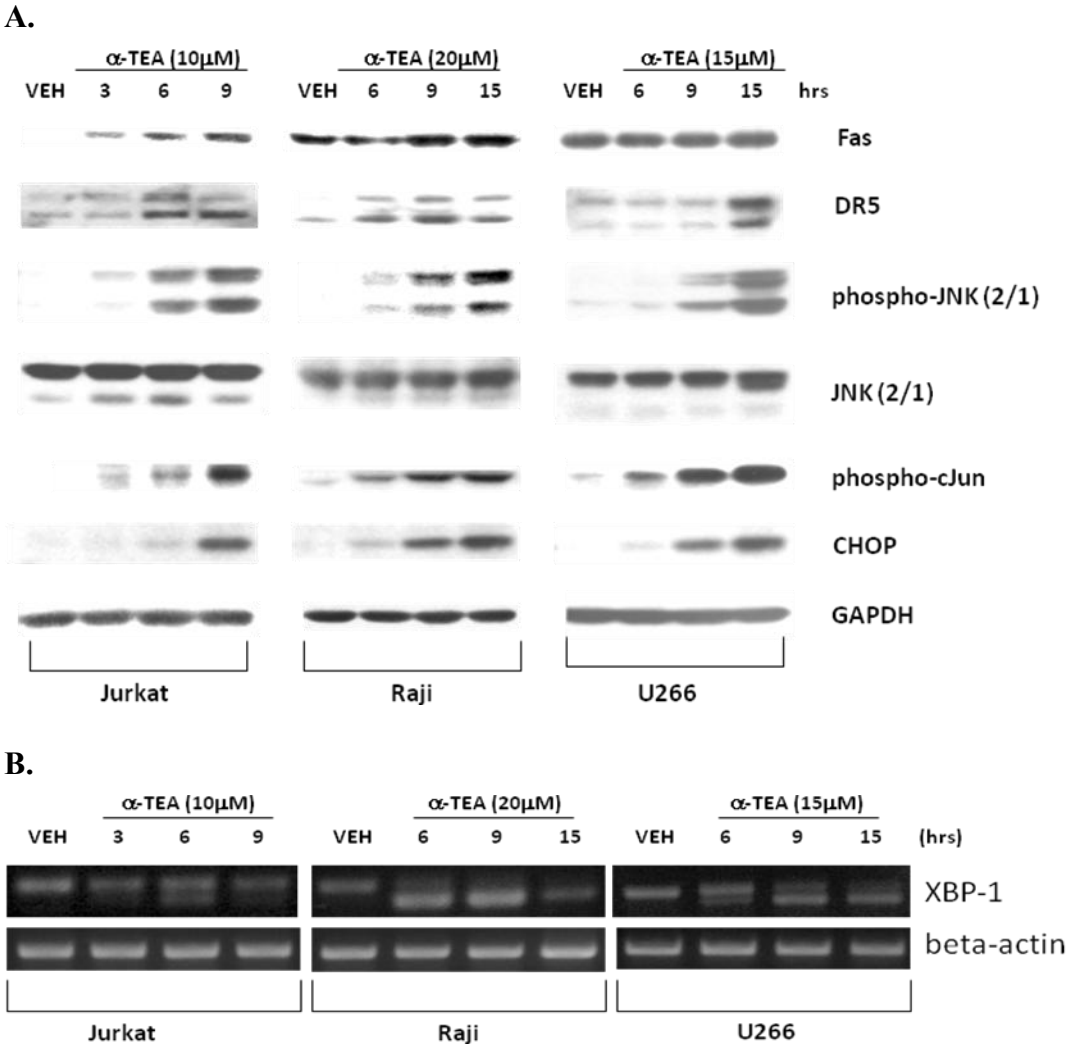

Figure 6. α-TEA upregulated death receptors, phospho-JNK and induced ER stress markers in Jurkat, Raji and U266 cells. Jurkat, Raji and U266 cells were treated with vehicle or α-TEA at indicated doses for various time periods. (A) Whole cell lysates were prepared to detect protein levels of Fas, DR5, JNK, cJun and CHOP by western blotting. GAPDH was used as a loading control. Data are representative of three independent experiments. (B) RT-PCR was performed to examine XBP-1 mRNA splicing status. β-actin was used as a loading control.

α-TEA Induced Apoptosis Was Inhibited By Acid Sphingomyelinase Inhibitor Desipramine

Previous studies suggested that the lipid second messenger ceramide plays an important role in inducing apoptosis. Recent work in our lab shows that ceramide is involved in α-TEA induced apoptosis in human breast cancer cells. Ceramide is generated in plasma membrane from sphingomyelin hydrolysis catalyzed by acid sphingomyelinase (aSMase). To determine if ceramide is involved in apoptosis induction by α-TEA in human hematological malignant cells, Jurkat, Raji and U266 cells were pre-treated with inhibitor of aSMase, desipramine, followed by α-TEA treatment (Figures 7 & 8). Apoptosis induced by α-TEA in Jurkat, Raji and U266 cells was inhibited by desipramine as indicated by reduced PARP cleavage (Figure 7A & B), and reduced caspase 9 in the three cell lines. Caspase 8 and tBid were reduced in Jurkat and U266 cells (Figure 7A). Moreover, desipramine partially blocked α-TEA-induced upregulation of JNK2/1 and p-cJun in the three cell lines. CHOP protein levels were reduced in Jurkat and Raji cells (Figure 8). These data suggest that ceramide generated from sphingomyelin hydrolysis may function as an upstream factor that is responsible for apoptosis induction by α-TEA in these cells.

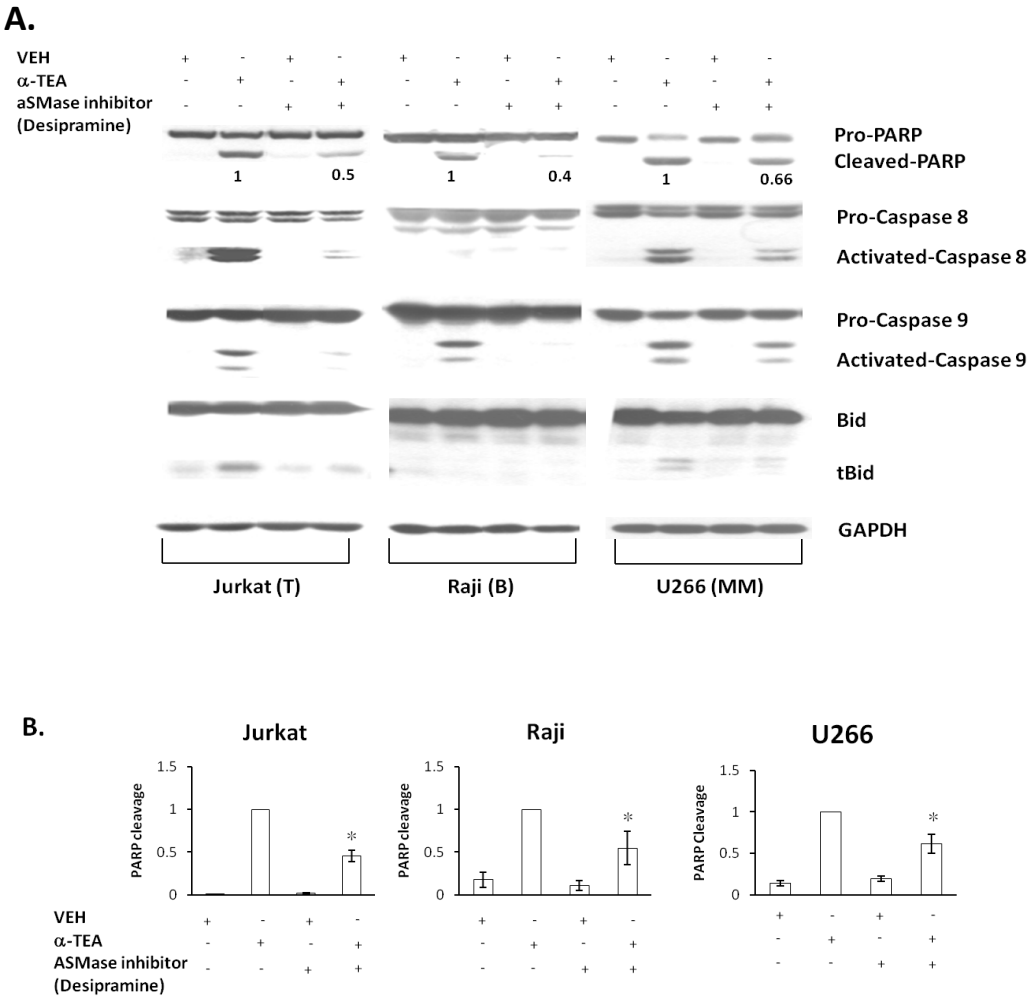

Figure 7. α-TEA induced apoptosis was inhibited by aSMase inhibitor desipramine. Jurkat, Raji and U266 cells were pretreated with vehicle or desipramine at 25 μM for 2 hours, followed by vehicle or α-TEA (10 μM in Jurkat, 20 μM in Raji, or 15 μM in U266) treatment for 9 hours. **(A)** Whole cell lysates were tested by western blotting for protein levels of PARP, caspase 8, caspase 9 and Bid. **(B)** Densitometric analysis of the PARP cleavage bands in western blotting. A data are representative of three independent experiments. B data are depicted as mean ± S.D. of three independent experiments. * = significantly different from vehicle control $P < 0.05$.

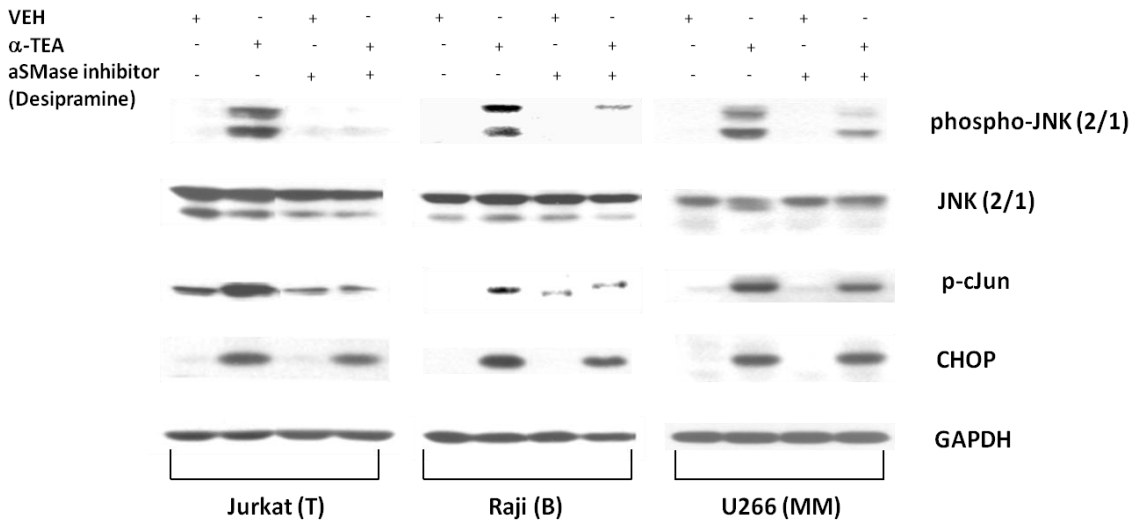

Figure 8. Desipramine partially blocked α-TEA-induced upregulation of phospho-JNK and CHOP. Jurkat, Raji and U266 cells were pretreated with vehicle or desipramine 25 μM for 2 hours, followed by vehicle or α-TEA (10 μM in Jurkat, 20 μM in Raji, or 15 μM in U266) treatment for 9 hours. Whole cell lysates were tested by western blotting for protein levels of phospho-JNK2/1, JNK 2/1, p-cJun and CHOP. GAPDH served as lane load controls. Data are representative of three independent experiments.

α-TEA Downregulated Anti-apoptotic Mediators in Jurkat, Raji and U266 Cells

To test the effects of α-TEA on anti-apoptotic signaling mediators in Jurkat, Raji and U266 cells, the three cells were treated with vehicle or α-TEA for various time periods, followed by western blotting of whole cell lysates for detection of anti-apoptotic protein levels (Figure 9). α-TEA downregulated the levels of phospho-IκBα in Raji and U266 cells, but not Jurkat cells, suggesting inhibition of the NF-κB signaling in Raji and U266 cells (Figure 9). Furthermore, antiapoptotic proteins XIAP (Raji and U266), cFLIP (Jurkat, Raji and U266), survivin (Raji and U266) and Bcl-2 (Raji) were downregulated by α-TEA (Figure 9).

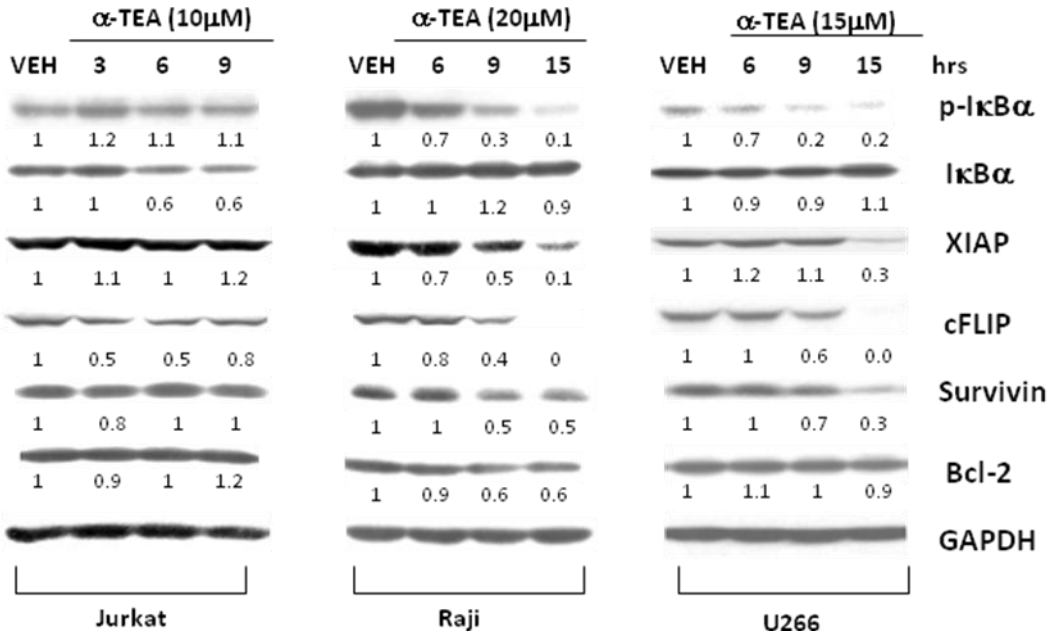

Figure 9. α-TEA downregulated anti-apoptotic factors in Jurkat, Raji and U266 cells. Jurkat, Raji and U266 cells were treated with vehicle or α-TEA at indicated doses for various time periods. Whole cell lysates were prepared to detect protein levels of a number of antiapoptotic proteins by western blotting. GAPDH was used as a loading control. Data are representative of three independent experiments.

THE APOPTOTIC EFFECTS OF DHA IN JURKAT AND U266 CELLS

To explore possible cooperative effects of α-TEA, the apoptotic-inducing properties of DHA were first tested in Jurkat and U266 cells. Jurkat and U266 cells were treated with DHA at 20-80 μM for 24 hours. Apoptosis was evaluated by annexin V-FITC/PI analysis (Figure 10). Data show that DHA significantly induced apoptosis in Jurkat and U266 cells in a dose dependent manner. Jurkat cells were more sensitive to DHA compared with U266 cells (Figure 10).

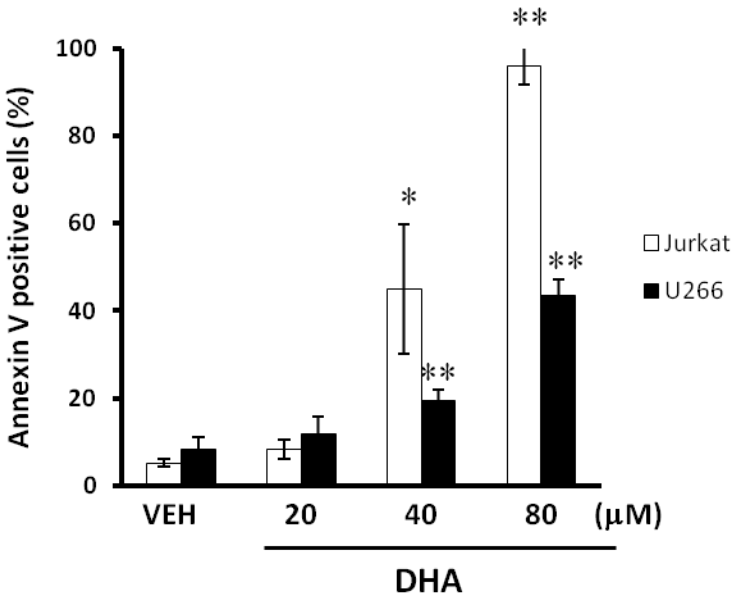

Figure 10. The apoptotic effects of DHA in Jurkat and U266 cells. Jurkat and U266 cells were treated with vehicle or DHA (20, 40, or 80 μM) for 24 hours. Apoptosis was evaluated by annexin V-FITC/PI analysis. Data are presented as the mean ± S.D. of three independent experiments. Student's *t* test was used to determine the statistical significance. *: $P < 0.05$ relative to vehicle treatment. **: $P < 0.01$ relative to vehicle treatment.

THE EFFECTS OF DHA ON ANTI- AND PRO-APOPTOTIC GENES IN JURKAT AND U266 CELLS

To further examine mediators involved in DHA induced apoptosis in Jurkat and U266 cells, both cell lines were treated with vehicle or DHA at various doses (Figures 11 and 12). DHA increased levels of cleaved PARP, p-IκBα, p-JNK 2/1, CHOP, and DR5, and reduced levels of anti-apoptotic mediators survivin and Cflip in Jurkat cells (Figure 11).

Treatment of U266 cells with 15, 30, or 60 μM DHA for 16 hours enhanced levels of cleaved PARP, phospho-JNK 2/1, CHOP and DR5, and reduced levels of anti-apoptotic mediators survivin and cFLIP (Figure 12).

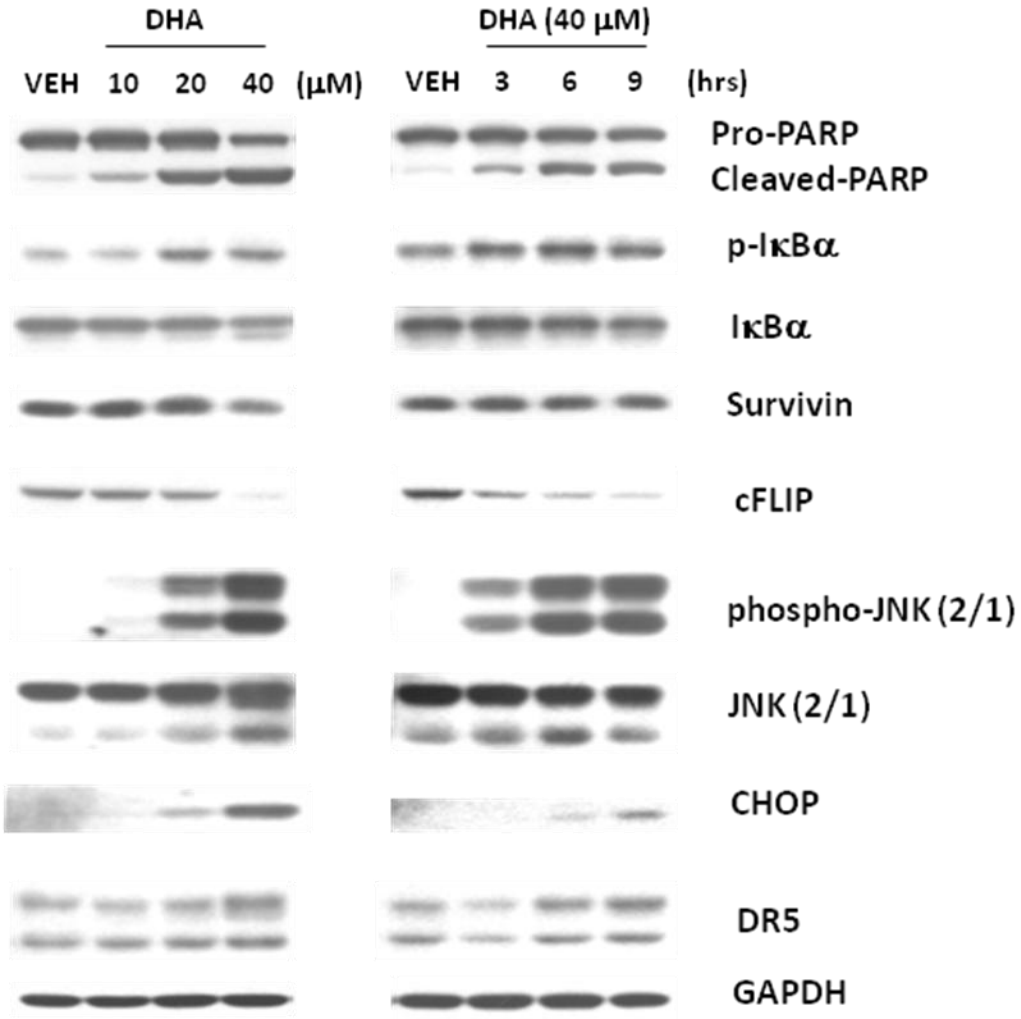

Figure 11. The effects of DHA on pro- and anti-apoptotic molecules in Jurkat cells. Jurkat cells were treated with vehicle or DHA (10, 20, or 40 μM) for 8 hours (left panel); or treated with vehicle or DHA 40 μM for 3, 6, or 9 hours. Western blot analyses were performed to detect protein levels of several anti- and pro-apoptotic genes. Data are representative of three independent experiments.

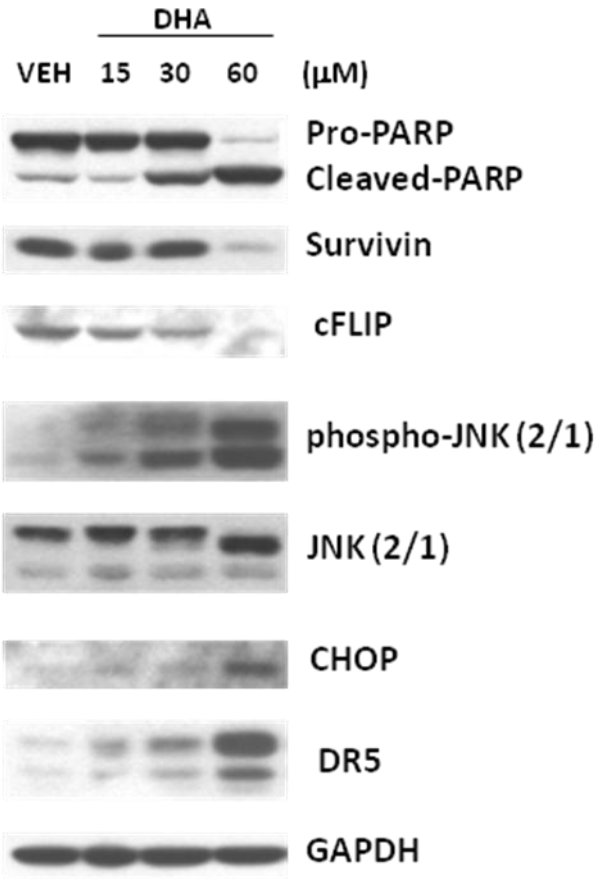

Figure 12. The effects of DHA on pro- and anti-apoptotic molecules in U266 cells. U266 cells were treated with vehicle or DHA (15, 30, or 60 μM) for 16 hours. Western blot analyses were performed to detect protein levels of several anti- and pro-apoptotic genes in these cells. Data are representative of two independent experiments.

COMBINATION EFFECTS OF α-TEA PLUS DHA IN JURKAT AND U266 CELLS

To examine the combination effects of α-TEA and DHA in human hematological malignant cells, Jurkat and U266 cells were treated with DHA (10 or 20 μM), α-TEA (2.5 or 5 μM) alone or together for 24 hours. Apoptosis was evaluated by annexin V-FITC/PI analysis (Figure 13). The combination of α-TEA plus DHA exhibited enhanced apoptotic effects in Jurkat cells, but not in U266 cells (Figure 13). The combination of α-TEA plus DHA acted cooperatively to enhance levels of cleaved PARP, p-IκBα, p-JNK 2/1, CHOP and DR5, and to reduce levels of cFLIP in Jurkat cells. Survivin levels were marginally reduced (Figure 14).

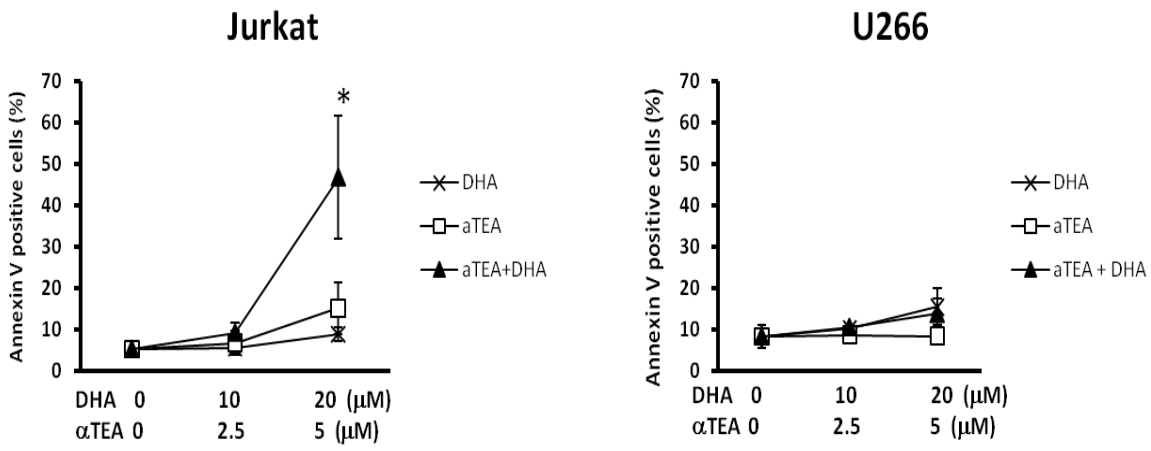

Figure 13. Combination apoptotic effects of α-TEA plus DHA in Jurkat and U266 cells. Jurkat and U266 cells were treated with DHA (10 or 20 μM), α-TEA (2.5 or 5 μM) alone or together for 24 hours. Apoptosis was evaluated by annexin V- FITC/PI flow cytometry analysis. Results represent mean ± SD of three independent experiments. Student's *t* test was used to determine the statistical significance. *: Significant difference from either α-TEA or DHA alone ($P < 0.05$).

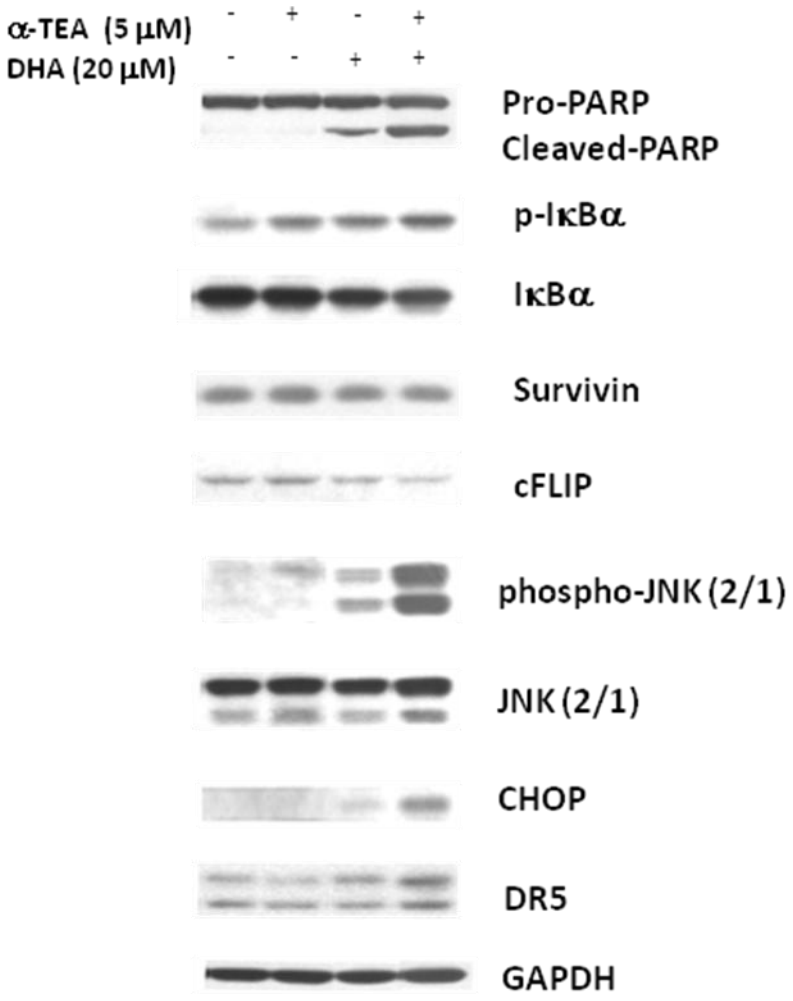

Figure 14. Combination effects of α-TEA plus DHA on anti- and pro-apoptotic proteins in Jurkat cells. Jurkat cells were treated with α-TEA (5 μM) or DHA (20 μM) alone or in combination for 9 hours. Western blot analyses were performed to detect protein levels of several anti- and pro-apoptotic mediators. Data are representative of two independent experiments.

Chapter 5: Discussion

Studies examined the ability of vitamin E analog α-TEA to induce apoptosis in human hematological malignant T cells (Jurkat), B cells (Raji), and multiple myeloma plasma cells (U266), omega-3 fatty acid DHA alone and in combination with α-TEA to induce apoptosis in Jurkat and U266 cells. α-TEA and DHA alone significantly induced apoptosis in the cell lines in comparison to control, and the combination of α-TEA and DHA acted cooperatively to induce apoptosis in Jurkat cells but not U266 cells. Jurkat cells were the most sensitive to α-TEA-induced apoptosis, and Raji cells were the least sensitive. Compared with human breast cancer cells, the hematological cells showed greater sensitivity to α-TEA-induced apoptosis (Yu et al., 2010; Tiwary et al., 2010). The high sensitivity of hematological malignant cells to α-TEA-induced apoptosis provides the scientific basis for using α-TEA as a treatment in hematological tumors, especially given the fact that hematological malignant cells occur in the blood and bone marrow where higher concentrations of α-TEA are easier to obtain.

α-TEA-induced apoptosis in hematological malignant cells was shown to be via extrinsic and intrinsic apoptotic pathways. α-TEA induced activation of caspase 8, 9 and 3 in Jurkat and U266 cells, and caspase 9 and 3 in Raji cells. In addition, Bid cleavage was induced in Jurkat and U266 cells. These data suggest that α-TEA-induced apoptosis in Jurkat and U266 cells is via caspase 8-dependent mitochondria pathway involving caspase 8 activation of tBid. However, the α-TEA-induced apoptosis in Raji cells is caspase 8-independent, involving only the intrinsic mitochondria pathway. Aspirin-induced apoptosis in Raji cells involves caspase 8, 9 and 3 activation (Pique et al., 2000). Fas ligand-induced apoptosis in Raji cells involves DISC formation and caspase 8 activation (Bando et al., 2003) On the other hand, CM1 (centrocyte/-blast marker 1)

induces apoptosis in Raji cells only by changing mitochondrial permeability, and Fas/caspase 8 is not involved (Kim et al., 2002). The fact that death receptor/caspase 8 signaling is not involved in α-TEA-induced apoptosis in Raji cells may explain why Raji cells are less sensitive to α-TEA-induced apoptosis in comparison with Jurkat and U266 cells.

Studies were conducted to determine if death receptors were involved in α-TEA induced apoptosis. α-TEA upregulated protein expression of death receptors Fas (Jurkat and Raji) and DR5, active form of JNK and cJun, and CHOP in these cells. These data suggest that death receptor/caspase 8 signaling is involved in α-TEA-induced apoptosis in Jurkat and U266 cells. Whereas, in Raji cells, death receptors may not be involved since data show that caspase 8 is not activated in these cells. JNK activation appears to have a role in the apoptosis in all the three cell lines. Studies also showed that ER stress was involved in α-TEA induction of apoptosis. ER stress marker CHOP was upregulated, and XBP-1 mRNA splicing was induced by α-TEA in all three lines, suggesting ER stress involvement. ER stress may mediate the apoptotic effect by activating JNK which in turn induce mitochondria-dependent apoptosis; or by increasing CHOP expression which induces DR5 expression (Kim et al., 2008; Yamaguchi, Wang, 2004).

To examine the possible involvement of ceramide in apoptosis induction by α-TEA, the aSMase inhibitor desipramine (inhibits ceramide formation) was used to pretreat these cells prior to α-TEA treatment. Desipramine partially blocked α-TEA-induced apoptosis in the three cell lines as indicated by reduced PARP cleavage. In addition, desipramine inhibited activation of caspases and Bid, as well as upregulation of proapoptotic molecules JNK and CHOP (Jurkat and Raji). These data suggest that ceramide generated from sphingomyelin hydrolysis by aSMase functions as an upstream apoptosis mediator in α-TEA-induced cell death. It has been reported that JNK plays a

critical role in ceramide induced apoptosis (Chen et al., 2008). Ceramide induces JNK phosphorylation, phospho-JNK activates Bim and induces apoptosis via the mitochondria-dependent pathway (Chen et al., 2008). Ceramide has also been reported to act upstream of ER stress to induce apoptosis (Sauane et al., 2010; Chen et al., 2008). This may explain the data showing reduced levels of CHOP in Jurkat and Raji cells pretreated with desipramine. This effect was not seen in U266 cells, suggesting de novo synthesis of ceramide may play a bigger role in U266 cells, or the interaction between ceramide and ER is cell specific. The inhibitory effects of desipramine on JNK activation and CHOP expression were not equivalent. While JNK activation was almost totally abolished by desipramine in Jurkat and Raji cells, elevated CHOP expression in these cells was only inhibited to a much less level. The same trend also happened in U266 cell, where JNK activation was inhibited while CHOP expression was not affected by desipramine. These results suggest that JNK activation in these cells in response to α-TEA is independent of ER stress.

In addition to the effects on proapoptotic genes, α-TEA also downregulated levels of antiapoptotic molecules cFLIP, survivin, Bcl-2, and XIAP. These alterations were correlated with downregulation of phospho-IκBα in Raji and U266 cells, suggesting NF-kB involvement. It has been reported that NF-κB signaling is constitutively activated in Raji and U266 cells, and inhibiting this signaling is sufficient to induce apoptosis (Malara et al., 2008; Zhang et al., 2008).

In exploring possible combination applications of α-TEA, DHA was tested separately and in combination with α-TEA in Jurkat and U266 cells. DHA induced apoptosis in Jurkat and U266 cells at 40 μM, which is lower than the physiologically relevant dose of DHA, 50 μM (Kim et al., 2010). Data also showed that the combination of α-TEA plus DHA acted cooperatively in induction of apoptosis in Jurkat cells, but not

in U266 cells. This enhanced apoptotic effect in Jurkat cells was correlated with enhanced upregulation of pro-apoptotic mediators DR5, phospho-JNK and CHOP, and downregulation of anti-apoptotic mediators cFLIP and survivin. Interestingly, phospho-IκBα in Jurkat cells was upregulated by α-TEA and DHA when tested alone, and this effect was enhanced with the combination treatment. Although NF-κB signaling is implicated in elevated proliferation and apoptosis resistance in many cancer cells, it also exhibits proapoptotic effects probably by inducing transcription of proapoptotic genes such as Fas and Fas ligand (Collett, Campbell, 2006). Another proposed mechanism for NF-κB to induce apoptosis is through specific inhibition of CDK4 activity which induces translocation of RelA from cytoplasm to nucleoplasm and then to nucleolus (Thoms et al., 2007). In addition, NF-κB is reported to mediate UV-induced apoptosis by promoting JNK activation (Liu et al., 2006). The role of NF-κB signaling in Jurkat cells in response to α-TEA and DHA requires further investigation.

Chapter 6: Summary and Future Studies

In summary, human hematological malignant Jurkat (T-lineage leukemia), Raji (B-lineage lymphoma) and U266 (multiple myeloma) cells exhibited high sensitivity to α-TEA induced apoptosis. There were common mechanisms mediating the apoptotic effects of α-TEA in the three cell lines, as well as cell type-specific mediators. Ceramide generated from sphingomyelin hydrolysis by aSMase appears to play an upstream role in the events involved in α-TEA-induced apoptosis in the three cell lines. Based on these studies, we propose that ceramide, as an upstream mediator, enhances α-TEA-induced apoptosis by activating JNK and ER stress. We propose that activated JNK involvement in α-TEA induced apoptosis is via activation Bim which translocates to mitochondria, triggering mitochondrial permeability transition. ER stress induces death receptor-dependent and mitochondria/caspase-dependent apoptotic pathways. Death receptor/caspase 8 signaling is involved in α-TEA induced apoptosis in Jurkat and U266 cells, but not in Raji cells. α-TEA down-regulation of anti-apoptotic genes XIAP, cFLIP and survivin enhances apoptosis by removing inhibitors of death receptor signaling. The relationship between ceramide generation and downregulation of these anti-apoptotic mediators require further study. Enhanced apoptotic actions of the combination of α-TEA plus DHA provide a potential new treatment for T lineage based leukemia.

Future studies include:

1. Further confirmation of the involvement of ER stress, death receptors, and JNK in α-TEA-induced apoptosis by conducting knock down studies using siRNA and chemical inhibitors.

2. Further examination of the involvement of NF-κB signaling pathway in α-TEA-induced apoptosis by conducting knock down and over-expression studies.

3. Determine the anticancer efficacy of α-TEA in xenograft models of hematological malignancies.

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